Mechanisms of LtxA (Leukotoxin), a Potent New Anti-Inflammatory Agent for the Treatment of Alopecia Areata

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Alopecia areata is an autoimmune condition where activated, pro-inflammatory white blood cells (WBCs) attack the hair follicles, resulting in hair loss. Migration of these activated WBCs from the blood stream and into the follicle tissue requires interaction between the integrin, lymphocyte function-associated antigen-1 (LFA-1) on WBCs, and ICAM-1 on vascular endothelial cells. High levels of active LFA-1 are uniquely expressed on WBCs that are involved in autoimmune and inflammatory conditions. The natural biologic agent LtxA (Leukothera) preferentially targets and depletes disease activated and malignant WBCs by binding to active LFA-1. The experimental drug has demonstrated significant therapeutic efficacy against autoimmune/inflammatory conditions such as psoriasis and allergic asthma in mouse models for these diseases. In addition, when injected into rodents, rhesus macaques, and dogs, LtxA was demonstrated to be physiologically active, biologically specific, and extremely well-tolerated. LFA-1 is an attractive target for therapy because it is only normally present on WBCs and has been shown to be activated and overexpressed on WBCs that are responsible for autoimmune/inflammatory conditions.

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Alopecia areata (AA) is a condition that is characterized by damage to the hair follicles and subsequent loss of hair (Hordinsky, 2013). Damage to the follicle tissue is caused by activated, pro-inflammatory white blood cells (WBCs) migrating into the hair follicles from the blood stream. Accumulation of WBCs in the follicle tissue of patients with AA has been described as a ''swarm of bees'' because of the appearance of the cells surrounding the follicles. These activated WBCs consist of at least T cells, NK cells, eosinophils, and mast cells (Elston *et al.*, 1997; Gilhar *et al.*, 1997; Wasserman *et al.*, 2007; Ito and Tokura, 2014; Santos *et al.*, 2015).

THE GREAT MIGRATION

Migration into and accumulation of the activated WBCs in the hair follicle requires the beta-2 integrin lymphocyte function-associated antigen-1 (LFA-1). LFA-1, composed of the two polypeptide chains CD11a and CD18, is present exclusively on the surface of WBCs and can exist is at least two functional states, an active and an inactive state (Hogg *et al.*, 1992; Kinashi, 2005). In the active state, LFA-1 assumes an ''exposed'' configuration where it can bind to its receptor, ICAM-1, on vascular endothelial cells. In contrast, when LFA-1 is in the inactive state, the molecule is tucked in and unable to bind to ICAM-1 (Carman and Springer, 2003). Binding of active LFA-1 to ICAM-1 signals the WBC to travel into the surrounding tissue (Hogg *et al.*, 1993; Giblin and Lemieux, 2006). The LFA-1/ICAM-1 interaction has an important role in the healthy immune response because it allows activated WBCs to be called upon by cytokines when needed during infection or injury. However, when LFA-1 is excessively active or overexpressed on the surface of WBCs, there is an accumulation of activated WBCs in the associated tissue, which can result in autoimmune disease and inflammatory disorders (Pals *et al.*, 1989; McMurray, 1996; Engelhardt, 2006), such as AA.

TARGETING THE IMPOSTERS

One therapeutic strategy to target the activated WBCs in autoimmune disease and inflammation is to use inhibitors of LFA-1, which block the binding of LFA-1 to ICAM-1 (Weitz-Schmidt *et al.*, 2001; Yusuf-Makagiansar *et al.*, 2002; Kvist *et al.*, 2008; Badell *et al.*, 2010; Pilat *et al.*, 2012). Blocking this LFA-1/ICAM-1 interaction prevents the migration of activated WBCs into the tissue from the blood stream. Thus, there is an accumulation of WBCs in the peripheral blood, also known as leukocytosis (Vugmeyster *et al.*, 2004; Koszik *et al.*, 2010). One drawback to this strategy is that once the blocking agent is depleted, the activated WBCs are free to bind ICAM-1 and migrate back into the tissue. Thus, patients need to be maintained on the drug continuously in order for the serum levels to remain high enough to have a pharmacological response. Indeed, the anti-LFA-1 monoclonal

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Abbreviations: AA, alopecia areata; LFA-1, lymphocyte function-associated antigen-1; WBC, white blood cell

antibody, efalizumab (Raptiva), was administered to patients weekly for treatment of psoriasis (Jullien *et al.*, 2004; Krueger *et al.*, 2008). Similarly, drugs that just block a component of the immune system, such as tumor necrosis factor-α inhibitors, are also given to patients on a regular basis for the treatment of autoimmune diseases and inflammatory disorders.

An alternate strategy for treating immune-mediated disorders is to deplete the cells that are involved in the condition. An advantage of this approach over the use of blocking agents is that frequent dosing is often not required since the disease-related cells are eliminated rather than just transiently and incompletely blocked. This depletion approach is also more robust because treatment eliminates the source of inflammation, and thereby targets an earlier step of the pathogenic process. An example of such a therapy is rituximab (rituxan), which depletes B cells and is approved for treating patients with rheumatoid arthritis (Goldblatt and Isenberg, 2008; Dorner *et al.*, 2009a, 2009b). Patients are maintained on rituximab by dosing them once every 6 months.

A new experimental therapeutic that is able to deplete a subset of WBCs is leukotoxin (LtxA; Leukothera). LtxA is a natural protein that is derived from a bacterium (*Aggregatibacter actinomycetemcomitans*) found in the mouth of humans (Kachlany, 2010). The protein has a natural specificity for WBCs because its receptor is LFA-1 (Lally *et al.*, 1997, 1999). Furthermore, we have shown that LtxA binds preferentially to the active form of LFA-1, thus eliminating only the most activated WBCs in the body, and not resting WBCs (Hioe *et al.*, 2011; Stenderup *et al.*, 2011; DiFranco *et al.*, 2012; Gupta *et al.*, 2015).

We have reported very significant efficacy and safety of LtxA in numerous animal models of disease. In humanized mouse models for leukemia (Kachlany *et al.*, 2010) and lymphoma (Difranco *et al.*, 2015), LtxA is able to eliminate the cancer with only a few doses, and the cancer never returns. We have also shown that LtxA is highly effective at treating psoriasis in a human xenograft mouse model (Stenderup *et al.*, 2011). LtxA caused more significant benefit than efalizumab for all parameters that were measured, including thickness of the skin, clinical psoriasis score, and lymphocyte infiltration. We recently published that LtxA also provides significant therapeutic benefit in a mouse model for allergic asthma (Gupta *et al.*, 2015). Mice that are administered house dust mite intranasally develop pulmonary inflammation and symptoms resembling allergic asthma. Administration of LtxA caused nearly complete reduction of the WBCs (monocytes) that infiltrated the lung tissue as well as a significant decrease in pulmonary inflammatory cytokines, and these effects were more pronounced than with dexamethasone, the most potent steroid that is clinically available (Gupta *et al.*, 2015).

When administered to rodents, dogs, and non-human primates, LtxA causes a very rapid (within minutes) drop in a subset of WBCs; and within a few hours, the WBC counts return to starting values (Kachlany *et al.*, 2010; DiFranco *et al.*, 2013). The replenishment of peripheral blood WBCs is likely due to the migration of cells from the tissue back into the blood stream. This effect provides yet another beneficial mechanism by which LtxA works. Therefore, in addition to eliminating the most activated, pro-inflammatory WBCs from the peripheral blood, LtxA also draws in the activated WBC population that resides in the tissue where they are causing the inflammation and damage. Importantly, severe adverse reactions have thus far not been observed in any of the species in which LtxA has been evaluated. Thus, because LtxA does not distinguish between cell type *per se*, but rather the activation state of LFA-1, the therapy eliminates all of the cell types implicated in disease; allowing a more comprehensive approach (Figure 1).

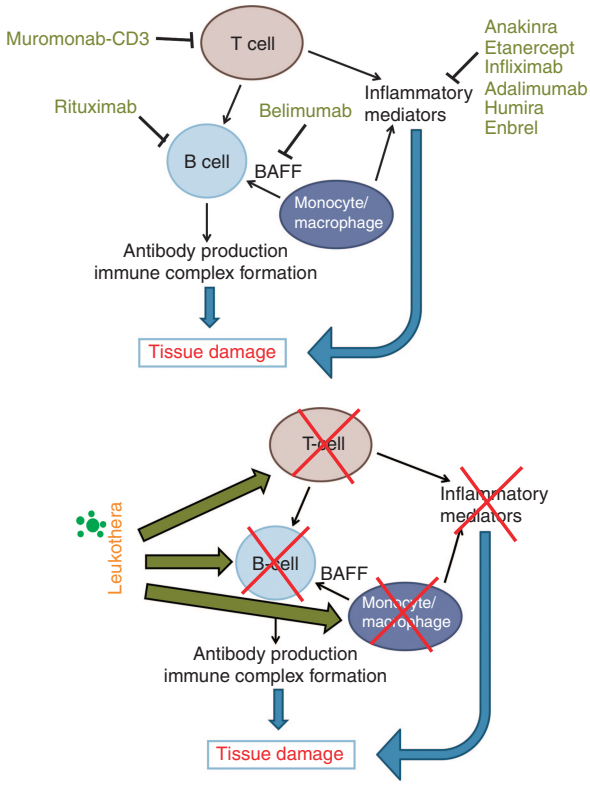

Figure 1. Current targeted therapies for WBC disorders affect a single component of the immune system, such as a cell type or a specific cytokine (top). Because the immune system is redundant, blocking only one component is not always the most effective strategy for treating autoimmune and inflammatory conditions, which are multifaceted. In contrast, LtxA (Leukothera) targets the WBCs that are highly activated and most relevant for disease (bottom). This approach eliminates the source of the cytokines, which are responsible for driving the inflammatory process.

While the immune system is clearly targeted by LtxA, there has been no evidence that LtxA causes detrimental immunosuppression mainly because (1) the agent is so selective that it targets only the activated component of the immune system and (2) the observed effects on WBCs are transient and reversible. Thus, LtxA only ''subdues'' the immune response while it eliminates those cells most relevant for disease. Indeed, targeting immune cells is inevitable if one wishes to treat a condition in which the immune system is the primary culprit.

MECHANISMS OF LtxA-MEDIATED CELL DEATH

Studies on the mechanism in which LtxA kills activated WBCs have resulted in numerous interesting and novel revelations. LtxA kills various cell types in different ways, depending on the machinery that is available in the cells. For example, the agent kills malignant and activated monocytes by binding to LFA-1, being internalized by vesicle formation, and then being transported to the lysosome where it causes lysosomal damage, rearrangement of the actin cytoskeleton, and raid cell death (DiFranco *et al.*, 2012; Kaur and Kachlany, 2014). LtxA can also activate the inflammasome in human monocytes, which results in the release of cytokines and pro-inflammatory cell death (Kelk *et al.*, 2011). In malignant and activated lymphocytes, in addition to LFA-1, LtxA requires the cell death receptor Fas (CD95) and caspase 8, but does not signal via the traditional FasL pathway (Difranco *et al.*, 2015). Activation of Fas then leads to the release of cytochrome c from the mitochondrial intermembrane space and activation of caspases 3, 7, and 9 (Lally *et al.*, 1999; Yamaguchi *et al.*, 2001). Hence, even within a single cell type, numerous pathways of cell death appear to be activated by LtxA, which ensures the terminal fate of the cell.

LtxA AND AA

An effective treatment for AA should ideally act rapidly and without significant side effects. LtxA can be administered intravenously or subcutaneously and would eliminate the ''rogue'' activated WBC population very efficiently from patients. It is not clear how frequently LtxA would have to be given to provide benefit, but based on its mechanisms of action and the dosing regimens of other drugs that deplete cells, it is likely that frequent dosing would not be required. In addition to systemic administration, local administration in the form of intradermal injections or topical formulations is also very possible with LtxA. Ultimately, clinical trials would be required to identify the ideal formulation and dosing schedule for treating AA with LtxA-based products.

FUTURE DIRECTIONS

To determine the potential applicability of LtxA for the treatment of AA, future studies should include both *in vitro* and *in vivo* experimentation. It would be interesting to determine if patients with AA have increased expression of the LFA-1 genes and proteins compared with non-affected individuals. With the availability of the gene expression data from these populations (Petukhova and Christiano, 2013; Betz *et al.*, 2015), this study is highly feasible. It would also be revealing to perform histopathological analysis with AA hair follicle tissue samples for detection of LFA-1 on the surface of the WBCs that constitute the ''swarm of bees''. This *in situ* data would demonstrate that the implicated WBCs could be targets for LtxA. Indeed, early evidence already suggests that LFA-1 is highly expressed on the cells that infiltrate the affected skin in AA patients (Ghersetich *et al.*, 1996). Finally, LtxA should be evaluated in the mouse model for AA that has recently been used successfully to study other therapeutics (Katikaneni *et al.*, 2013; Xing *et al.*, 2014). Positive results in one or more of these studies would allow the continued development of LtxA as a potent and safe anti-inflammatory therapy for the treatment of AA.

CONFLICT OF INTEREST

SCK is founder of Actinobac Biomed, Inc., a company that has licensed the use of LtxA as a therapeutic agent from Rutgers University. SCK has equity in the company and receives a consulting fee.

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